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13. ABSTRACT (Maximum 200 Words) One of the principal objectives of our laboratory is to understand the mechanisms by which hormones and growth factors regulate normal mammary gland development and how these same regulatory pathways become altered in breast cancer. Specific emphasis has been placed upon studying the mechanisms by which the lactogenic hormones, prolactin, hydrocortisone and insulin, regulate milk protein gene expression. The CCAAT/enhancer binding protein β (C/EBP β), Yin Yang-1 (YY-1), signal transducers and activators of transcription 5 (STAT5), and the glucocorticoid receptor (GR) all have been shown to mediate the hormonal and developmental regulation of β -casein gene expression. However, the mechanism by which these transcription factors as well as coactivator proteins coordinately function to promote normal mammary gland development and breast cancer remains undefined. To understand the role of C/EBP β , STAT5, GR and comodulatory factors in hormonally-regulated chromatin remodeling at the β -casein promoter and enhancer, we have initiated chromatin immunoprecipitation assays in HC11 cells and in the mammary gland and liver from lactating mice. In addition, we have analyzed mammary gland development in several specific mouse knockouts. The examination of mammary transplants from GR ^{-/-} and CBP ^{+/-} mice suggested that MR may compensate for the loss of GR in mammary gland development during pregnancy, lactation and involution, and that haploinsufficiency of CBP does not appear to affect mammary gland development.				
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Introduction

One of the principal objectives of our laboratory is to understand the mechanisms by which hormones and growth factors regulate normal mammary gland development and how these same regulatory pathways become altered in breast cancer. Ectopic expression of hormones such as prolactin by breast tumors has been implicated in tumor progression and a poor prognostic outcome in many patients with breast cancer. Therefore, specific interest has been placed upon studying the mechanisms by which the lactogenic hormones, prolactin, hydrocortisone and insulin, regulate milk protein gene expression and how the alterations in the levels of these hormones may function in the growth promotion of breast tumors. CCAAT/enhancer binding protein β (C/EBP β), Yin Yang-1 (YY-1), signal transducers and activators of transcription 5 (STAT5), and the glucocorticoid receptor (GR) have been shown to mediate the hormonal and developmental regulation of a particular milk protein gene, β -casein. However, the mechanism by which these transcription factors as well as coactivator proteins coordinately function to promote normal mammary gland development and breast cancer remains undefined. Therefore, we are interested in studying the mechanisms by which the lactogenic hormones and local growth factors regulate milk protein gene (β -casein) expression through a specific and ordered assembly of transcriptional factors and comodulatory proteins at the proximal promoter and distal enhancer.

Body

Objective 1: To understand the role of C/EBP β , STAT5, GR and comodulatory factors in hormonally-regulated chromatin remodeling at the β -casein promoter and enhancer.

1a. RNA analysis

Hormone dependent gene transcription was assessed in order to evaluate the activation of β -casein gene in HC11 cells treated with prolactin for various periods of time. In these experiments total RNA was isolated from non-treated cells and cells treated with prolactin for 30 min, 2 hrs, 6 hrs, and 24 hrs; and 5 μ g of RNA was reverse transcribed using a RT-PCR kit. PCR reactions were performed with primers designed to exon VII region of β -casein gene. As expected, we observed that transcripts were slightly induced within 30 min after stimulation with prolactin and β -casein mRNA levels continued to increase for 24 hrs. These experiments were performed in parallel to ChIP assays and served as a basic control.

1b. Chromatin immunoprecipitation assays in HC11 cells

Controls

Role of Stat5a in activation of β -casein gene

Initially, we modified a few steps from published protocols in order to perform ChIP assays in HC11 mammary epithelial cells. These modifications were as follows:

Although complete cell lysis is not absolutely necessary (and difficult to achieve), it is very important that lysis be as efficient as possible. Efficient lysis is important to obtain a reproducible amount of cell breakage among different samples to reliably compare results from different treatments and time points. We found that after cross-linking with formaldehyde, the treatment of HC11 cells with 10mM DTT helped facilitate cell lysis. Sonication conditions were also optimized. To reduce non-specific binding chromatin was pre-cleared with pre-immune serum. This step helped to decrease the "no antibody" control significantly and improved the specific to non-specific signal.

A number of primer sets were designed to specifically amplify different regions at the β -casein gene proximal promoter, distal enhancer, and exon VII (utilized in ChIP PCR as a negative control). Mouse GAPDH primers also were designed as a positive control for a constitutively expressed gene and used successfully for PCR amplification. Then using these modifications and reagents, we demonstrated the chromatin remodeling at the promoter and enhancer of the β -casein gene in ChIP assays using anti-acetylated histone H3 and H4 antibodies. In addition, we wanted to determine if there were kinetic differences in the assembly of specific transcription factors such as Stat5a, GR and C/EBP β at the proximal promoter and distal enhancer. Using a carboxy-terminal antiStat5a antibody, we were able to demonstrate that within 30 min of stimulation of HC11 cells with prolactin Stat5a binding to the β -casein promoter increased, followed by a decrease to a basal level that remained constant up to 24 hrs following hormone stimulation. Different binding kinetics were observed at the distal enhancer of the β -casein gene, which contains three Stat5 sites. Within 30 min of prolactin stimulation we again detected an increase in binding, but in this case the level appeared not to change significantly with time following hormone treatment. These results suggest that Stat5 may be important for transcriptional initiation, but other proteins (such as C/EBP β , NF1's etc.) are necessary for long-term maintenance of the transcription complex. The recruitment of co-activators is also under investigation. Quantitation of these results will be accomplished by the use of real time PCR assays, which are currently under development for each of the primer sets.

1c. Chromatin immunoprecipitation assays in mouse lactating mammary gland

We have also extended these studies in cell culture to optimize the ChIP assay for mammary gland tissue from lactating mice, in order to validate the results obtained in HC11 cell model *in vivo*. As a negative control for β -casein gene expression we will analyze chromatin isolated from the livers of the same animal. The main difficulty, which had to be overcome, was how to disaggregate the mammary epithelial cell nuclei from the fatty stroma in lactating tissue after cross-linking with formaldehyde. Dounce homogenization alone did not work, so we employed a Polytron homogenizer. Sonication conditions also were optimized. The remainder of the protocol was similar to that performed in HC11 cells. In preliminary ChIP experiments using an anti-acetylated histone H3 antibody we have been able to detect a histone acetylation at both proximal promoter and distal enhancer of β -casein gene. This result was confirmed using GAPDH primers for PCR amplification. Further studies will analyze the chromatin expression of the albumin enhancer and promoter in liver and the lactating mammary gland and

changes in the β -casein promoter and enhancer in mammary epithelial cells during development.

Objective 2: To examine the roles of the transcriptional regulatory GR and CBP on ductal morphogenesis, lobuloalveolar development, and functional differentiation of the mammary gland.

2a. Collect and examine glands from various time points of mid-pregnancy, day 1 lactation and days 1-4 involution in the GR secondary transplants and perform proliferation and TINEL assays and immunohistochemistry.

To study the role of GR at different stages of mammary gland development, mammary glands were rescued from GR^{-/-} mice by transplantation into the cleared fat pad of wild type mice. In virgin mice GR^{-/-} outgrowths displayed abnormal ductal morphogenesis characterized by distended lumina, multiple layers of luminal epithelial cells in some regions along the ducts and increased periductal stroma. In contrast, the loss of GR did not result in overt phenotypic changes in mammary gland development during pregnancy, lactation and involution. Surprisingly, despite the known synergism between glucocorticoids and prolactin in the regulation of milk protein gene expression, WAP and β -casein mRNA levels were unaffected in GR^{-/-} transplants as compared to wild type transplants. That the mineralocorticoid receptor (MR) might compensate for the loss of GR was suggested by the detection of MR in the mammary gland at day one of lactation. This hypothesis was tested using explant cultures derived from the GR^{-/-} transplants in which the mineralocorticoid fludrocortisone was able to synergistically induce β -casein gene expression in the presence of prolactin and insulin. These studies suggest that MR may compensate for the absence of GR at some, but not at all stages of mammary gland development. This hypothesis needs to be tested using conditional alleles for both GR and MR currently being developed by our collaborator Dr. Gunter Schütz's laboratory in Heidelberg.

2b. Examine CBP heterozygous female mice for lactation deficiency.

2c. Perform transplants with CBP heterozygous mice and examine phenotype at different stages of mammary gland development including virgin, pregnancy, lactation and involution.

We received CBP^{+/-} animals from Dr. Andrew Kung based on his original observations that CBP^{+/-} females failed to nurse their pups. We were interested in the role of CBP in the expression of milk protein genes such as WAP and β -casein and hypothesized that the lactation defect in the CBP^{+/-} females could be caused by the failure to properly undergo mammary gland differentiation and/or produce milk. Due to the CBP^{+/-} animals' low fertility rate and high *in utero* mortality rate (mating CBP^{+/-} males with wild type females, 1 in 10 pups were CBP^{+/-} and only 1 in 4 of those pups were female), we decided to do the experiments that were proposed in my fellowship concurrently. We performed mammary gland transplantation experiments where CBP^{+/-} virgin mammary tissue was transplanted into one side of the cleared fat pad of 3-week old wild type recipients and age-matched wild type virgin mammary tissue was

transplanted into the opposite side of the recipient. At the same time, we examined the mammary glands of CBP^{+/-} females at various stages of development. CBP^{+/-} transplanted tissue was morphologically indistinguishable from the wild type transplants at every stage of mammary gland differentiation from virgin through day one lactation. We are currently performing northern blot analysis of this tissue at day one lactation, specifically looking for changes in the expression of the milk proteins, WAP and β -casein. Surprisingly, when we examined the mammary glands of CBP^{+/-} animals, morphologically, the tissue appeared the same as wild type. It was observed, however, that the milk produced at day one lactation had the consistency of yogurt and it is likely that the pups were unable to suckle this thick milk. We are performing northern blot analysis of this tissue as well. Additionally, consecutive matings of both transplanted animals and CBP^{+/-} animals did not result in mammary gland hyperplasia, as originally hypothesized, because of the increased lymphoid tumors seen in these animals. Until the northern blot analysis is performed, we cannot conclude that milk protein expression is not affected by the dosage of CBP in the mammary gland. However, it does appear that the dosage of CBP does not affect the development of the mammary gland.

Key Research Accomplishments

The ChIP assay was developed and optimized for HC11 cells and successfully extended for lactating mammary gland tissue and liver.

Stat5a activation by prolactin appears to initiate transcription of β -casein gene, but most likely requires cooperation with other transcription factors to maintain transcriptional activation.

Loss of GR resulted in abnormal ductal morphogenesis in virgin mice, but did not cause overt phenotypes in mammary gland development during pregnancy, lactation and involution.

MR may compensate for the absence of GR at some promoters, and surprisingly WAP and β -casein mRNA levels were unaffected in GR^{-/-} as compared to wild type transplants.

Haploinsufficiency of CBP does not appear to affect the development of the mammary gland when assessed in mammary transplants, and, therefore, any effects seen in CBP heterozygotes on lactation are most likely due to systemic rather than direct effects on the mammary epithelium.

Reportable Outcomes

Manuscripts:

Kingsley-Kallesen M, Mukhopadhyay SS, Wyszomierski SL, Schanler S, Schutz G and Rosen JM. The mineralocorticoid receptor may compensate for the loss of the glucocorticoid receptor at specific stages of mammary gland development.

Funding applied for, based on work supported by this award:

The studies presented in this report were included as part of a competitive renewal of NIH grant CA16303 to support one specific aim. This grant was funded and was designated an NIH MERIT award. These funds will be help supplement costs associated with this project.

Conclusions

By doing a kinetic analysis of casein mRNA accumulation coupled with ChIP assays in HC11 cells following lactogenic hormone induction, and in the mammary gland and liver, we hope to be able to better understand the role of C/EBP β , STAT5, GR and comodulatory factors in hormonally-regulated chromatin remodeling at the β -casein promoter and enhancer. So far, we have developed the ChIP assay and optimized its use in HC11 cells, as well as the mammary gland and liver from lactating mice. Preliminary data indicate Stat5 may be important for prolactin-induced transcriptional initiation, but that other transcription factors, such as C/EBP β , GR and NFI's may be necessary for long-term maintenance of the transcription complex. We are in the process of confirming and quantitating these results by the use of real time PCR assays and will also continue employing the ChIP assay to examine the recruitment of co-activators to the β -casein enhancer and promoter.

Additionally, the examination of mammary gland transplants from GR^{-/-} mice suggested that MR may compensate for the loss of GR at certain stages of mammary gland development, such as during pregnancy, lactation and involution. Our study of transplants from CBP^{+/-} mice indicated that haploinsufficiency of CBP does not appear to affect the development of the mammary gland, and that the influence of CBP heterozygosity previously observed on lactation most likely was the result of systemic, rather than direct effects in the mammary epithelium.

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